p-Chloroamphetamine Induces Serotonin Release through Serotonin Transporters†

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ABSTRACT: *p*-Chloroamphetamine (PCA) interacts with serotonin transporters in two membrane vesicle model systems by competing with serotonin for transport and stimulating efflux of accumulated serotonin. In plasma membrane vesicles isolated from human platelets, PCA competes with [^{3}H]imipramine for binding to the serotonin transporter with a K_D of 310 nM and competitively inhibits serotonin transport with a K_I of 4.8 nM. [^{3}H]Serotonin efflux from plasma membrane vesicles is stimulated by PCA in a Na^+-dependent and imipramine-sensitive manner characteristic of transporter-mediated exchange. In membrane vesicles isolated from bovine adrenal chromaffin granules, PCA competitively inhibits ATP-dependent [^{3}H]serotonin accumulation with a K_I of 1.7 μM and, at higher concentrations, stimulates efflux of accumulated [^{3}H]serotonin. Stimulation of vesicular [^{3}H]serotonin efflux is due in part to dissipation of the transmembrane pH difference (ΔpH) generated by ATP hydrolysis. Part of PCA's ability to stimulate efflux may be due to its transport by the vesicular amine transporter. Flow dialysis experiments demonstrated uptake of [^{3}H]PCA into chromaffin granule membrane vesicles in response to the ΔpH generated in the presence of Mg^{2+} and ATP. In plasma membrane vesicles, no accumulation was observed using an NaCl gradient as the driving force. We conclude that rapid nonmediated efflux of transported PCA prevents accumulation unless PCA is trapped inside by a low internal pH.

Amphetamine and its derivatives are indirectly acting sympathomimetic amines which are believed to exert their effects by releasing endogenous biogenic amines from nerve terminals. Certain amphetamine derivatives, including *p*-chloroamphetamine (PCA),[1] fenfluramine, 3,4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxymethamphetamine (MDMA), preferentially release serotonin both in vivo (Fuller et al., 1965; Pletscher et al., 1963) and in vitro (Johnson et al., 1986; Nichols et al., 1982; Schmidt et al., 1987). Two observations suggest that serotonin transport systems mediate the serotonin release induced by these compounds: (1) Ca^{2+} is not required for amphetamine-induced serotonin release (Johnson et al., 1986), suggesting that exocytosis is not involved, and (2) inhibitors of serotonin transport block the effect of these amphetamine derivatives. Thus, it is likely that amphetamines may induce release by reversal of the transport systems which normally accumulate serotonin to high levels within the neuron and the synaptic vesicle.

In addition to releasing serotonin, amphetamine derivatives also lead to a long-term depletion of serotonin (Clinschmidt et al., 1976; Ricaurte et al., 1985) which correlates with morphological damage to serotonergic nerve endings (Mamounas & Molliver, 1988; Molliver & Molliver, 1990; O'Hearn et al., 1988; Ricaurte et al., 1985). The serotonin transporter has been implicated in this neurotoxicity since inhibitors of serotonin transport block the destruction of serotonergic terminals (Fuller et al., 1975; Ross & Froden, 1977; Schmidt et al., 1987). These results suggest that the serotonin transporter either mediates the entry of neurotoxic amphetamines into serotonergic terminals or participates in sequelae leading to serotonin release and depletion (Fuller, 1980) or both. Serotonin release may be only one of many processes contributing to neurotoxicity. Recently, evidence has accumulated implicating dopamine release in the action of neurotoxic amphetamines (Johnson & Nichols, 1991; Schmidt et al., 1985).

Although transport systems have been implicated in the action of amphetamine derivatives, little is known about specific interactions between amphetamine derivatives and serotonin transporters. Serotonergic neurons, like other cells which secrete serotonin, contain two serotonin transport systems which function in series (Rudnick et al., 1980). One of these systems transports serotonin into the cell; the other sequesters intracellular serotonin within secretory vesicles.

Membrane vesicles consisting of purified platelet plasma membrane contain a Na^+-dependent, imipramine-sensitive serotonin transporter (Rudnick, 1977). When appropriate transmembrane ion gradients are imposed, these vesicles accumulate [^{3}H]serotonin to concentrations several hundredfold higher than in the external medium. Transport requires external Na^+ and Cl^-, and is stimulated by internal K^+ (Keyes & Rudnick, 1982; Nelson & Rudnick, 1982; Talvenheimo et al., 1983). Results from a variety of experiments suggest that the transporter moves serotonin with Na^+ and Cl^- across the membrane in one step of the reaction and moves K^+ in the opposite direction in a second step (Rudnick, 1986). cDNA clones coding for serotonin transporters have recently been isolated from rat brain (Blakely et al., 1991) and rat basophilic leukemia cells (Hoffman et al., 1991). The sequence homology between these cDNAs strongly suggests that identical proteins are responsible for serotonin reuptake into both peripheral blood cells and presynaptic nerve endings and that platelets provide a sound model for neuronal serotonin reuptake.

Membrane vesicles isolated from bovine adrenal chromaffin granules accumulate biogenic amines, including serotonin, by a coupled transport system driven by ATP hydrolysis (Njus et al., 1986). Apparently, the same system is responsible for amine uptake into secretory vesicles from platelets, mast cells, and nerve endings (Fishkes & Rudnick, 1982; Maron et al., 1979; Mota et al., 1954). The system consists of two

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[1] Abbreviations: PCA, *p*-chloroamphetamine; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane pH difference.

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components: an ATP-driven H^+ pump, the vacuolar AT-Pase, which acidifies the vesicle interior and also generates a transmembrane electrical potential ($\Delta\psi$, interior positive) (Dean et al., 1986); and the reserpine-sensitive vesicular amine transporter, which couples efflux of one or more H^+ ions to the uptake of each molecule of biogenic amine (Knoth et al., 1981).

Recent evidence from this laboratory suggests that MDMA is a substrate for the plasma membrane serotonin transporter and exchanges with serotonin (Rudnick & Wall, 1992a,b). In the present work, we have extended these results by examining the interaction of PCA with the plasma membrane and vesicular serotonin transporters. The results suggest that PCA is a substrate for both transport systems and imply that PCA causes serotonin release by stimulating serotonin/PCA exchange.

EXPERIMENTAL PROCEDURES

Membrane Vesicles. Outdated human platelet concentrates were purchased from the Connecticut Red Cross. Platelets from 50–100 individuals were pooled for each membrane preparation. Platelet plasma membrane vesicles were isolated by the method of Barber and Jamieson (1970) with the modifications described previously (Rudnick & Nelson, 1978). Chromaffin granule membrane vesicles were prepared as described by Schuldiner et al. (1978) by repeated osmotic lysis of bovine adrenal medullary chromaffin granules isolated by differential sedimentation.

Transport Assays. (A) Filtration. Transport of [^{3}H]serotonin and [^{3}H]PCA into plasma membrane vesicles was measured at 25 °C using the previously described filtration assay (Rudnick, 1977). Unless indicated otherwise, vesicles were equilibrated with 10 mM lithium phosphate buffer, pH 6.7, containing 133 mM K_2SO_4 and 1 mM $MgSO_4$, and diluted into an external medium consisting of 0.2 M NaCl containing 10 mM lithium phosphate buffer, pH 6.7, 1 mM $MgSO_4$, and 0.1 μM [^{3}H]serotonin (12.3 Ci/mmol) or 0.1 μM [^{3}H]PCA (17.9 Ci/mmol). Chromaffin granule membrane vesicles were diluted to a concentration of approximately 0.25 mg/mL in 0.3 M sucrose containing 10 mM K-Hepes, pH 8.5, 5 mM KCl, 2.5 mM $MgSO_4$, 5 mM Na_2-ATP, and 0.1 μM [^{3}H]-serotonin or 0.1 μM [^{3}H]PCA (unless indicated otherwise). Reactions (200 μL per assay) were stopped by dilution, filtration, and washing, and filtered vesicles were counted as described previously (Schuldiner et al., 1978). The standard error of replicate assay values was typically less than 5% of the mean.

(B) Flow Dialysis. [^{3}H]Serotonin and [^{3}H]PCA accumulation by chromaffin granule membrane vesicles was determined using flow dialysis, as described by Colowick and Womack (1969), using a cell with a 200-μL upper chamber separated from a 50-μL lower chamber by a cellulose dialysis membrane with an average pore radius of 2.4 nm (VWR Scientific). The upper chamber was stirred constantly by a small magnetic stirring bar. Control rates of dialysis were determined by placing 0.2 mL of a solution of [^{3}H]PCA or [^{3}H]serotonin in the upper chamber and pumping degassed solution through the lower chamber at a rate of 1 mL/min; 0.5-mL fractions were collected, and the radioactivity in the lower chamber effluent was measured by liquid scintillation counting.

Serotonin Efflux and Exchange. Platelet plasma membrane vesicles equilibrated with 10 mM lithium phosphate buffer, pH 6.7, containing 60 mM NaCl, 93 mM K_2SO_4, and 1 mM $MgSO_4$ were diluted 30-fold into 0.2 M NaCl containing 10 mM lithium phosphate buffer, pH 6.7, and 0.1 μM [^{3}H]-serotonin at 25 °C. After serotonin accumulation had reached a maximum (5–10 min), efflux was initiated by diluting the suspension 40-fold with the indicated medium. Thirty seconds after dilution, the vesicles were collected by filtering the suspension through Gelman GN-6 nitrocellulose filters. The reaction tube and filter were rapidly rinsed with 2 mL of ice-cold 0.2 M NaCl, and the filter was counted in 3 mL of Optifluor (Packard, Downers Grove, IL). Efflux from chromaffin granule membrane vesicles was measured in the same way, except that preloading was carried out in 0.3 M sucrose containing 10 mM K-Hepes, pH 8.5, 5 mM KCl, 2.5 mM $MgSO_4$, 5 mM Na_2-ATP, and 0.1 μM [^{3}H]serotonin. Efflux was measured after dilution into the same medium without ATP, $MgSO_4$, or [^{3}H]serotonin. The standard error of replicate assay values was typically less than 10% of the mean.

Imipramine Binding. Imipramine binding was measured at 25 °C using the filtration assay described previously (Humphreys et al., 1988). Membrane vesicles were suspended at a protein concentration of 0.3 mg/mL in an assay buffer of 200 mM NaCl containig 10 mM lithium phosphate, pH 6.7, 1 mM $MgSO_4$, and the indicated concentration of [^{3}H]imipramine (19–23 cpm/fmol). After a 20-min incubation, the reactions (300 μL per assay) were terminated by dilution with 4 mL of ice-cold isoosmotic NaCl and filtered through Whatman GF/B filters pretreated with 0.3% poly(ethylenimine). The tube and filter were washed 3 times with 4 mL of ice-cold NaCl solution. Filters were placed in Optifluor and counted after 5 h. Binding in the absence of Na^+ or in the presence of 100 μM serotonin was taken as a control for nonspecific binding. The standard error of replicate assay values was typically less than 5% of the mean.

ΔpH Measurements. Chromaffin granule membrane vesicles (80 μg of protein) were incubated at room temperature in a cuvette containing 2 mL of 10 mM Tris–EPPS (pH 8.5), 150 mM KCl, 6 μM acridine orange, and 5 mM ATP. The relative fluorescence of the mixture was measured using an excitation wavelength of 490 nm and an emission wavelength of 526 nm on a Perkin-Elmer LS-5B luminescence spectrometer. When the base-line fluorescence stabilized, acidification was initiated by the addition of 6 mM $MgSO_4$, and the relative fluorescence was monitored. Approximately 15 min later, additions of PCA were made, and the increase in fluorescence was measured. After the last addition, NH_4Cl was added to a final concentration of 10 mM to neutralize the vesicle interior and completely reverse the quenching. In replicate experiments, the PCA concentration required for half-maximal reversal of the fluorescence quenching varied by less than 10% of the maen value.

Synthesis of [^{3}H]PCA. *p*-Chlorophenyl acetone was synthesized from *p*-chlorophenylacetic acid by the procedure of King and McMillan (1951). The product, which was characterized by NMR, was reduced with NaB^3H_4 (NEN) in the presence of ammonia to yield β-[^{3}H]-*p*-chloroamphetamine. The reaction mixture was applied in aliquots of 100 μL to a 25 × 0.46 cm Ultrasphere ODS C-18 column (Beckman, San Ramon, CA) and eluted with 25% CH_3CN/75% 0.2 M ammonium formate and 0.1% triethylamine (adjusted to pH 3.0 with formic acid). [^{3}H]PCA coeluted with authentic PCA. The [^{3}H]PCA-containing fractions were pooled and concentrated in vacuo. The final specific radioactivity was calculated from the specific activity of the starting NaB^3H_4 to be 17.9 Ci/mmol. This [^{3}H]PCA was equipotent with authentic PCA for inhibition of imipramine binding to platelet plasma membranes (data not shown).

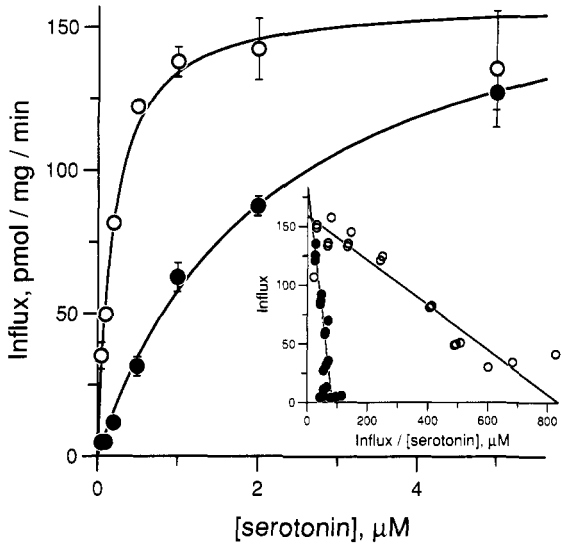

FIGURE 1: Competitive inhibition of plasma membrane serotonin transport by PCA. Rates of serotonin influx were measured in 20-s incubations as described under Experimental Procedures, using the indicated concentrations of [^{3}H]serotonin in the presence (filled circles) and absence (open circles) of 50 nM PCA. Serotonin concentration was varied by addition of unlabeled substrate. Uptake due to nonspecific processes, measured in the presence of 1 μM imipramine, was substracted from each measurement. Nonlinear regression analysis of the data gave a K_M of 0.164 ● 0.027 μM and a V_{max} of 152 ± 6 pmol mg^{-1} min^{-1} for the control and in the presence of 50 nM PCA a K_M of 2.16 ± 0.26 μM and a V_{max} of 180 ± 10 pmol mg^{-1} min^{-1}. Error bars indicate standard deviation (where it exceeds the symbol size) of the mean of triplicate measurements. Inset: The same data are plotted by the method of Hofstee (1952).

Protein Determination. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Materials. [^{3}H]Serotonin (12.6 Ci/mmol), NaB^{3}H$_4$, and [^{3}H]imipramine (40.4 Ci/mmol) were purchased from New England Nuclear. The (+) and (−) isomers of PCA were generous gifts from Dr. R. W. Fuller, Lilly Research Laboratories. All other reagents were reagent grade, purchased from commercial sources.

RESULTS

Plasma Membrane Vesicles. p-Chloroamphetamine (PCA) is a potent inhibitor of serotonin transport into plasma membrane vesicles. The data in Figure 1 demonstrate that PCA competitively inhibits the initial rate of [^{3}H]serotonin transport. This inhibition is overcome by high concentrations of substrate. In the inset, an Eadie–Hofstee plot, the V_{max} (y intercept) is unchanged by 50 nM PCA, but the K_M (−slope) is increased. From the extent of the increase, we estimate that the K_I for inhibition is 4.8 ± 0.58 nM for PCA. In separate experiments (not shown), the reversible nature of PCA inhibition was demonstrated. Transport into vesicles was inhibited 78% by incubation with 67 nM PCA, but sedimenting and resuspending the vesicles in fresh buffer reversed over 80% of the inhibition. PCA also competitively inhibits [^{3}H]-imipramine binding. The Scatchard plot shown in Figure 2 demonstrates that the total number of sites (B_{max} given by the x intercept) is unchanged by 1 μM PCA while the K_D for binding (given by the negative reciprocal of the slope) is increased. From these data, we calculated a K_D of 310 nM for PCA.

To test for the ability of PCA to exchange directly with serotonin, we allowed membrane vesicles containing 60 mM NaCl to accumulate [^{3}H]serotonin and then diluted them

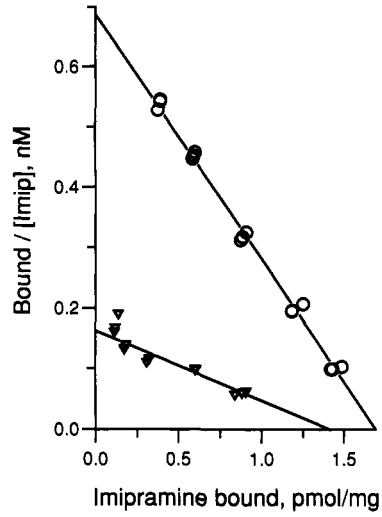

FIGURE 2: Competitive inhibition of imipramine binding by PCA. Imipramine binding to platelet plasma membrane vesicles was measured over a range of [^{3}H]imipramine concentrations in the presence (triangles) and absence (circles) of 1 μM PCA. Nonspecific binding, measured in the presence of 100 μM serotonin, was subtracted from each measurement. Nonlinear regression analysis of the data gave a K_D of 2.47 ± 0.08 nM and a B_{max} of 1.69 ± 0.02 pmol/mg for the control and in the presence of 1 μM PCA a K_D of 8.69 ± 0.68 nM and a B_{max} of 1.41 ± 0.06 pmol/mg. The data are plotted according to Scatchard (1949).

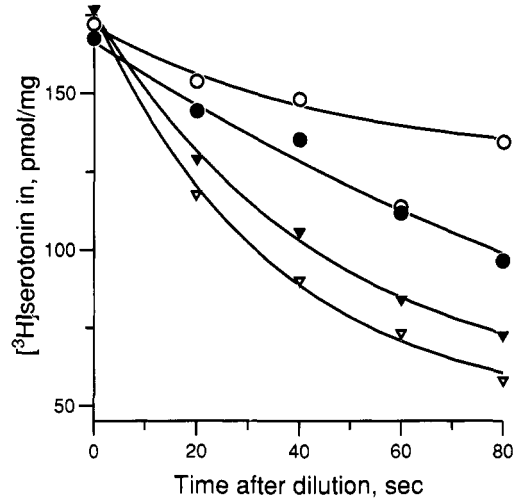

FIGURE 3: Time course of [^{3}H]serotonin efflux from plasma membrane vesicles. Vesicles preloaded with [^{3}H]serotonin as described under Experimental Procedures were diluted into 0.2 M NaCl containing 1 mM $MgSO_4$/10 mM lithium phosphate buffer, pH 6.7 (control, open circles), or the same medium containing 1 μM serotonin (5-HT, filled circles) or 1 μM PCA (PCA, filled triangles). Alternatively, veiscles were diluted into 0.17 M LiCl containing 30 mM KCl and 1 mM $MgSO_4$/10 mM lithium phosphate buffer, pH 6.7 mM (K^+, open triangles). At the indicated times, a portion of the diluted vesicle suspension was filtered and washed as described under Experimental Procedures. Data are from a typical experiment. At least three repeats of this experiment gave similar results.

40-fold into medium free of [^{3}H]serotonin. As shown in Figure 3, rapid loss of radiolabel occurred from these vesicles when the dilution medium contained 30 mM K^+ (open triangles). This efflux represents reversal of the normal transport reaction. Removal of K^+ from the dilution medium inhibited efflux (open circles) by decreasing the rate of transporter reorientation (Nelson & Rudnick, 1979), which becomes rate-determining under these conditions. Addition of 1 μM unlabeled serotonin increased efflux by a process of exchange (filled circles), which bypasses the slow step of transporter reorientation (Nelson & Rudnick, 1979). Addition of 10 μM

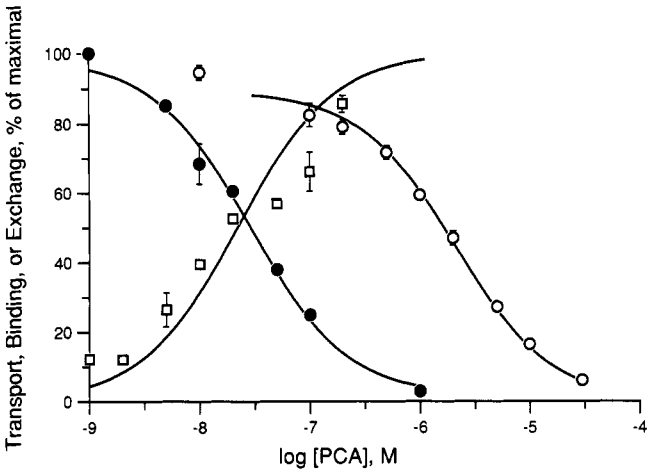

FIGURE 4: Effect of PCA on serotonin transport, imipramine binding, and serotonin efflux by plasma membrane vesicles. Transport (filled circles): PCA was added at the indicated concentration to the serotonin transport reaction performed as described under Experimental Procedures. The control rate of transport was 100.1 pmol mg^{-1} min^{-1} in the absence of added PCA. Half-maximal inhibition of transport occurred at 26.6 nM. The serotonin concentration used, 0.1 μM, is below the K_M, 0.17 μM. Binding (open circles): PCA was added at the indicated concentration to the imipramine binding reaction performed as described under Experimental Procedures with 1 nM [^{3}H]imipramine, a concentration below the K_D, 2.43 nM. The control binding under these conditions was 0.328 pmol/mg in the absence of added PCA. Half-maximal inhibition of binding occurred at 1.96 μM in this experiment. Efflux (squares): Vesicles which had accumulated [^{3}H]serotonin were diluted into NaCl medium containing PCA at the indicated concentration, and efflux was measured for 1 min as described under Experimental Procedures. The maximal extent of efflux was 56% in 1 min at 1 μM PCA. Half-maximal stimulation of efflux occurred at 56.1 nM. In separate experiments (not shown), serotonin half-maximally stimulated efflux at 34 ± 6 nM. Each point represents the average of triplicate determinations. Error bars represent the standard deviation and are plotted only where they exceeded the size of the symbol.

PCA stimulated loss of radiolabel even more than serotonin (filled triangles), suggesting that PCA is a substrate for the transporter that can replace serotonin. In separate experiments (not shown), the maximal effect of serotonin and PCA on [^{3}H]serotonin release was found to be identical.

During transporter-mediated serotonin efflux, internal [^{3}H]-serotonin must bind to the transporter within the vesicle lumen and dissociate to the external medium. According to our previously proposed mechanism for serotonin transport (Nelson & Rudnick, 1979), each time the transporter releases a molecule of [^{3}H]serotonin to the medium, it must undergo a slow conformational reorientation before binding another molecule of internal [^{3}H]serotonin. This slow step is accelerated directly by external K^+, which is concomitantly transported inside. The results described above suggest that PCA, as well as unlabeled serotonin, bypasses the slow step by allowing reversal of the relatively fast substrate transport step.

In support of this role for PCA, the concentration dependence for PCA stimulation of serotonin efflux (squares, Figure 4) is similar to that for its inhibition of serotonin influx (filled circles). Transport is inhibited over 50% at 50 nM and over 97% at 1 μM. Figure 4 also shows that inhibition of equilibrium [^{3}H]imipramine binding requires higher concentrations (open circles). Thus, the K_D for binding inhibition (310 nM, Figure 2) is 64-fold higher than the K_I for transport inhibition (4.8 nM, Figure 1). Also for serotonin, the K_D [1.7 μM (Talvenheimo et al., 1979)] is higher than the K_M for transport (0.16 μM, Figure 1), an observation attributed to rate-determining steps that follow serotonin translocation in the transport cycle (Nelson & Rudnick, 1979).

Table I: [Na^+] Dependence and Imipramine Sensitivity of Serotonin/PCA Exchange[a]

	efflux, % of internal contents		
treatment	no addition	serotonin	PCA
(1) control	7.7 ± 4.1	30.0 ± 2.6	40.2 ± 2.2
(2) K_2SO_4 in	4.2 ± 5.4	9.7 ± 4.2	10.0 ± 2.5
(3) LiCl out	− 3.6 ± 2.3	5.5 ± 4.5	11.3 ± 2.0
(4) 2 μM imipramine	11.9 ± 5.3	14.1 ± 5.4	17.8 ± 6.5

[a] Platelet plasma membrane vesicles equilibrated as described under Experimental Procedures with medium containing 60 mM NaCl (rows 1, 3, and 4) or with isoosmotic K_2SO_4 (row 2) were loaded with [^{3}H]serotonin and diluted into the following media: rows 1 and 2, 0.2 M NaCl containing 10 mM lithium phosphate buffer, pH 6.7; row 3, NaCl was replaced with equimolar LiCl; row 4, 2 μM imipramine was added to control medium. All values represent the percent [^{3}H]serotonin efflux in the first 30 s after dilution. In the absence of internal NaCl (row 2), the vesicles contained 180 pmol of serotonin per milligram of membrane protein (approximately 18 μM) at the time of dilution. In the presence of internal NaCl (rows 1, 3, and 4), this value decreased to 65 pmol/mg or 6.5 μM. Control efflux in medium containing 30 mM K^+ was 44.6 ± 5.4% efflux in 30 s.

To test the possibility that efflux stimulation results from nonspecific effects, such as increasing the membrane's permeability to serotonin, we measured the [Na^+] dependence and imipramine sensitivity of the PCA effect. The results in Table I show the effect of serotonin and PCA on increasing [^{3}H]serotonin efflux. The control (row 1) demonstrates efflux under conditions where NaCl is present both inside and outside the vesicles. Previous work suggests that efflux mediated by the serotonin transporter requires internal Na^+ and Cl^- as cotransported ions (Nelson & Rudnick, 1979, 1982). Vesicles in which K_2SO_4 replaced internal NaCl did not lose [^{3}H]serotonin in response to external unlabeled serotonin or PCA (Table I, row 2). According to the proposed mechanism for exchange (Nelson & Rudnick, 1979), stimulation by external substrate requires cotransport with external Na^+. Table I (row 3) shows that removal of external Na^+ accelerates efflux, as previously reported (Nelson & Rudnick, 1979). In the absence of external Na^+, however, neither PCA nor serotonin further stimulates efflux. Moreover, imipramine, which binds tightly to the serotonin transporter and prevents it from catalyzing serotonin translocation, also blocks the ability of PCA and serotonin to stimulate [^{3}H]serotonin efflux (Table I, row 4). These results imply that efflux is mediated by the serotonin transporter. Additional evidence for a specific action of PCA at the serotonin transporter is presented in Table II, where the (+) stereoisomer of PCA is more active than the (−) isomer for inhibition of serotonin transport and imipramine binding and stimulation of [^{3}H]-serotonin efflux.

Storage Vesicles. PCA also inhibits the ability of chromaffin granule membrane vesicles to accumulate [^{3}H]serotonin. Figure 5 demonstrates competitive inhibition by PCA of the initial rate of serotonin influx. As the concentration of serotonin is increased, the inhibition by PCA is less dramatic, as evidenced by the shallower slope in this Dixon plot. From the point of intersection of the three $1/v$ vs [PCA] curves, we estimated the K_I for transport inhibition by PCA to be 1.7 μM.

We measured efflux of [^{3}H]serotonin from chromaffin granule membrane vesicles induced by unlabeled serotonin, PCA, and the ionophore nigericin. When vesicles preincubated with ATP and [^{3}H]serotonin were diluted 40-fold into medium free of ATP and [^{3}H]serotonin, slow efflux of ra-

Table II: Stereoselectivity of PCA Effects on the Serotonin Transporter[a]

transport	pmol mg^{-1} min^{-1}	% inhibn
control	30.2 ± 1.5	
200 nM (+)PCA	14.5 ± 0.4	51.9 ± 1.4
200 nM (−)PCA	21.1 ± 0.5	30.2 ± 1.8
efflux	**% of total/min**	**% of control**
control (30 mM K_{out})	58.2 ± 2.0	
100 nM (+)PCA	26.5 ± 2.0	45.5 ± 3.5
100 nM (−)PCA	17.6 ± 1.4	30.3 ± 2.4
imipramine binding	**pmol/mg**	**% inhibn**
control	0.274 ± 0.007	
3 μM (+)PCA	0.016 ± 0.002	94.0 ± 0.6
3 μM (−)PCA	0.069 ± 0.002	74.8 ± 0.7

[a] Transport, efflux, and binding were measured as described under Experimental Procedures in the presence of the indicated concentration of PCA where indicated. Transport rates were measured in 20-s incubations, efflux conditions were as described in the legend to Table I, and binding was as in Figure 4.

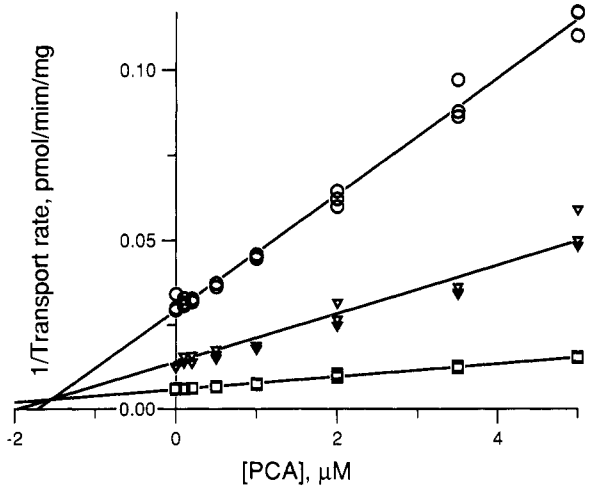

FIGURE 5: Dixon plot of vesicular transport inhibition by PCA. Rates of serotonin transport into chromaffin granule membrane vesicles were measured in 5-min incubations as described under Experimental Procedures, using 0.1, 0.3, and 1.0 μM [^{3}H]serotonin (circles, triangles, and squares, respectively) in the presence of the indicated concentrations of PCA. Uptake due to nonspecific processes, measured in the presence of 2.5 μM reserpine, was subtracted from each measurement. The K_I for PCA calculated from these data is 1.7 ± 0.2 μM.

diolabel ensued (Figure 6, open circles), which was markedly accelerated by PCA (open squares). External serotonin (filled circles) was much less effective at stimulating efflux than PCA, resulting in <20% efflux in 5 min compared to 60% efflux at the same time with the same concentration (200 μM) of PCA. Maron et al. (1983) showed that amine efflux catalyzed by the vesicular amine transporter is highly dependent on internal pH. Nigericin, in the presence of external K^+, catalyzes H^+/K^+ exchange which alkalinizes acidic vesicles. Under these conditions, serotonin efflux was extremely rapid, with less than 25% remaining inside after 5 min (filled squares).

Stimulation of serotonin efflux, and inhibition of its uptake, could reflect PCA's ability to be transported by the vesicular amine transporter. However, PCA, as a weakly basic amine, can also dissipate, by passive nonionic diffusion, the ΔpH which drives transport. To evaluate these possibilities, we measured the ability of PCA to dissipate ΔpH as measured by acridine orange fluorescence quenching. Figure 7 shows the trace from a typical experiment where quenching of acridine orange fluorescence was used to monitor internal

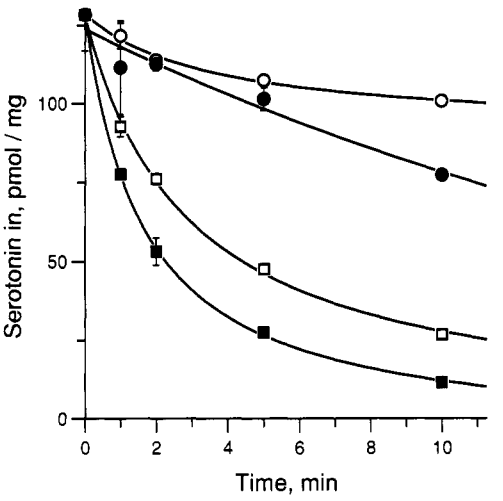

FIGURE 6: Time course of [^{3}H]serotonin efflux from chromaffin granule membrane vesicles. Vesicles preloaded with [^{3}H]serotonin as described under Experimental Procedures were diluted 40-fold into 0.27 M sucrose containing 10 mM K-EPPS, pH 8.5, 2.5 mM $MgSO_4$, 5 mM KCl, and 10 mM K_2SO_4 (control, open circles) or the same medium containing 200 μM serotonin (5-HT, filled circles), 200 μM PCA (PCA, open squares), or 1 μM nigericin (filled squares). At the indicated times, a portion of the diluted vesicle suspension was filtered and washed as described under Experimental Procedures. Each point represents the average of duplicates. Error bars represent the standard deviation and are plotted only where they exceeded the size of the symbol.

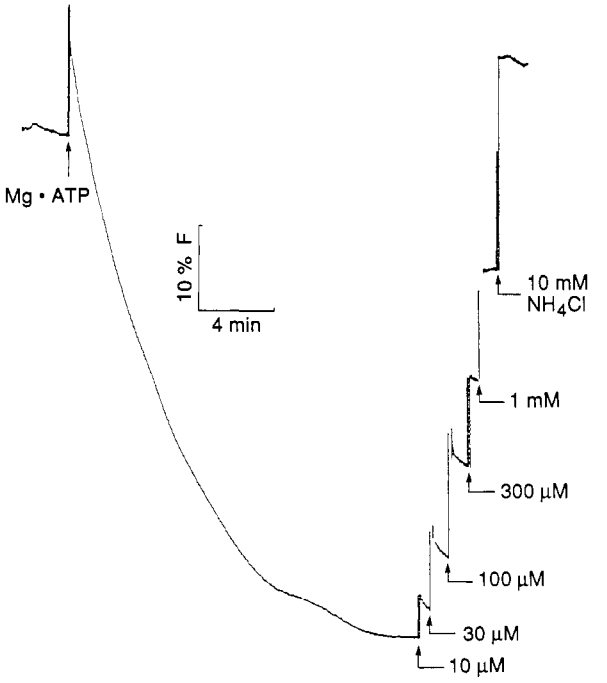

FIGURE 7: Dissipation by PCA of ATP-driven fluorescence quenching. Acridine orange quenching due to generation of ΔpH was measured as described under Experimental Procedures. Acidification was initiated by addition of $MgSO_4$ at the indicated time. PCA additions were made at the times and concentrations indicated, followed by 10 mM NH_4Cl.

acidification. When Mg^{2+} was added to vesicles in the presence of acridine orange and ATP, the fluorescence decreased, consistent with accumulation of the weakly basic fluorophore within the acidifying vesicle and concomitant concentration quenching (Rottenberg, 1979). On addition of increasing concentrations of PCA (shown by an arrow for each concentration in Figure 7), the fluorescence increased in discrete steps, consistent with dissipation of the ΔpH. At the end of

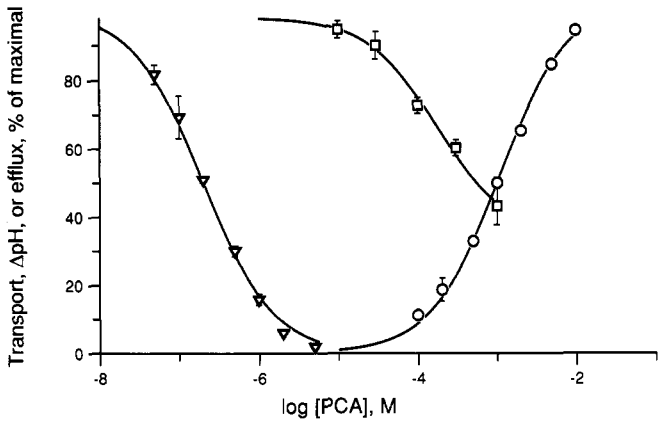

FIGURE 8: ΔpH and serotonin flux by chromaffin granule membrane vesicles. Transport (triangles): PCA was added at the indicated concentration to the serotonin transport reaction performed as described under Experimental Procedures. The control rate of transport was 28 pmol mg^{-1} min^{-1} in the absence of added PCA. The serotonin concentration used, 0.1 μM, is below the K_M, 4.3 μM (Kanner et al., 1979). Half-maximal inhibition of transport required 0.19 μM PCA. Efflux (circles): Vesicles which had accumulated [^{3}H]-serotonin were diluted into 0.3 M sucrose medium containing 10 mM K-EPPS, pH 8.5, 2.5 mM $MgSO_4$, 5 mM KCl, and PCA at the indicated concentration, and efflux was measured for 1 min as described under Experimental Procedures. The maximal extent of efflux was 94% in 1 min at 10 mM PCA, and half-maximal stimulation required 1.25 mM PCA. ΔpH dissipation (squares): Reversal of acridine orange quenching was determined at various PCA concentrations as shown in Figure 7 and described under Experimental Procedures. Half-maximal dissipation of ΔpH required approximately 0.18 mM PCA. Each point represents the average of triplicate determinations for the transport and efflux measurements, and duplicates for the ΔpH measurements. Error bars represent the standard deviation and are plotted only where they exceeded the size of the symbol.

the experiment, 10 mM NH_4Cl was added, which raised the internal pH to the preacidification level.

Figure 8 shows the concentration dependence for PCA inhibition of [^{3}H]serotonin transport, dissipation of ΔpH, and stimulation of [^{3}H]serotonin efflux in chromaffin granule membrane vesicles. In contrast with its effect in the plasma membrane system, PCA, at concentrations that inhibited transport (triangles), had little effect on efflux (circles) or ΔpH (squares). Moreover, at concentrations which stimulated efflux, PCA also caused noticeable dissipation of ΔpH. PCA was more potent than NH_4Cl at stimulating efflux or dissipating ΔpH, with a half-maximal activity at 30 μM, compared to 1 mM for NH_4Cl.

Transport of [^{3}H]PCA. To assess directly the ability of serotonin transporters to transport PCA, we synthesized [^{3}H]-PCA and tested it in transport assays. Using a filtration assay in which membranes are collected and washed on nitrocellulose filters, significant amounts of [^{3}H]PCA were not associated with either platelet plasma membrane vesicles or chromaffin granule membrane vesicles while, by the same assay, [^{3}H]-serotonin accumulation was robust (data not shown). We observed no imipramine-sensitive [^{3}H]PCA transport into platelet plasma membrane vesicles and no reserpine-sensitive [^{3}H]PCA transport into chromaffin granule membrane vesicles. Since the ability of PCA to dissipate ΔpH suggested that it was highly permeant through lipid bilayers, we attempted to measure [^{3}H]PCA accumulation by flow dialysis. This technique allows continuous monitoring of free radiolabel concentration in a dialysis chamber by measuring the rate at which radioactivity crosses a dialysis membrane in contact with that chamber.

The results shown in Figure 9 demonstrate ATP-dependent accumulation of both [^{3}H]serotonin and [^{3}H]PCA by chromaffin granule membrane vesicles (left panels). The initial rise in radioactivity followed addition of [^{3}H]serotonin (upper left) or [^{3}H]PCA (lower left) to the dialysis chamber containing membrane vesicles. After 5 min, ATP was added, leading to a decrease in the free concentration of both [^{3}H]-serotonin and [^{3}H]PCA as they were accumulated within vesicles. At 25 or 30 min, 20 mM NH_4Cl was added, and as the accumulated substrates leaked from the vesicle, their free concentration increased back toward the initial level. It is worth noting that the fractional decrease in free [^{3}H]serotonin concentration was significantly greater than that for [^{3}H]PCA, indicating a higher internal concentration for serotonin. In separate experiments (not shown), reserpine failed to inhibit ATP-dependent [^{3}H]PCA accumulation into chromaffin granule membrane vesicles.

In contrast, flow dialysis measurements detect [^{3}H]serotonin but not [^{3}H]PCA accumulation by plasma membrane vesicles (right panels). After initial addition of [^{3}H]serotonin (upper right) or [^{3}H]PCA (lower right) to the dialysis chamber containing NaCl solution, a small volume of a concentrated vesicle suspension equilibrated in K_2SO_4 was added, imposing transmembrane gradients of Na^+, Cl^-, and K^+. In the presence of 1 μM imipramine (filled circles), this addition resulted in a small decrease in the concentration of free radiolabel due to dilution and possibly some binding to the membranes. In the absence of imipramine (open circles), an additional decrease in [^{3}H]serotonin is apparent due to accumulation within the vesicles, but no such decrease was observed with [^{3}H]PCA.

DISCUSSION

It is generally accepted that biogenic amine transport systems mediate the amphetamine-induced release of catecholamines and serotonin from nerve terminals (Azzaro et al., 1974; Bonisch, 1984; Fischer & Cho, 1979). Studies with synaptosomes and brain slices show that release by amphetamines is independent of Ca^{2+} and is sensitive to transport inhibitors. Because of the limitations of these experimental systems, however, the detailed mechanisms by which amphetamines interact with plasma membrane and vesicular transport systems have not previously been elucidated. In particular, little is known about the interaction of serotonin transporters with those amphetamine derivatives, such as *p*-chloroamphetamine (PCA), which release serotonin.

The results presented here suggest that PCA is a substrate for the plasma membrane serotonin transporter. PCA competes with imipramine for binding sites and competes with serotonin for transport (Figures 1 and 2). The ability of PCA to stimulate Na^+-dependent and imipramine-sensitive serotonin release (Figure 3, Table I) is consistent with PCA transport by the serotonin transporter, resulting in serotonin/PCA exchange, and would not be expected for a nontransported inhibitor. Our data do not rule out the possibility that PCA, by binding to the serotonin transporter in a Na^+- and Cl^--dependent and imipramine-sensitive manner, activates the transporter to catalyze uncoupled serotonin efflux. The requirements for internal Na^+ and Cl^- (Table I) in this process and the similarity to serotonin exchange (Figure 3, Table 1) argue against uncoupling as a mechanism.

Much higher concentrations of PCA are required to inhibit imipramine binding at equilibrium relative to the concentration that inhibits transport or stimulates efflux (Figure 4). Rather than indicating two separate sites of action, this difference

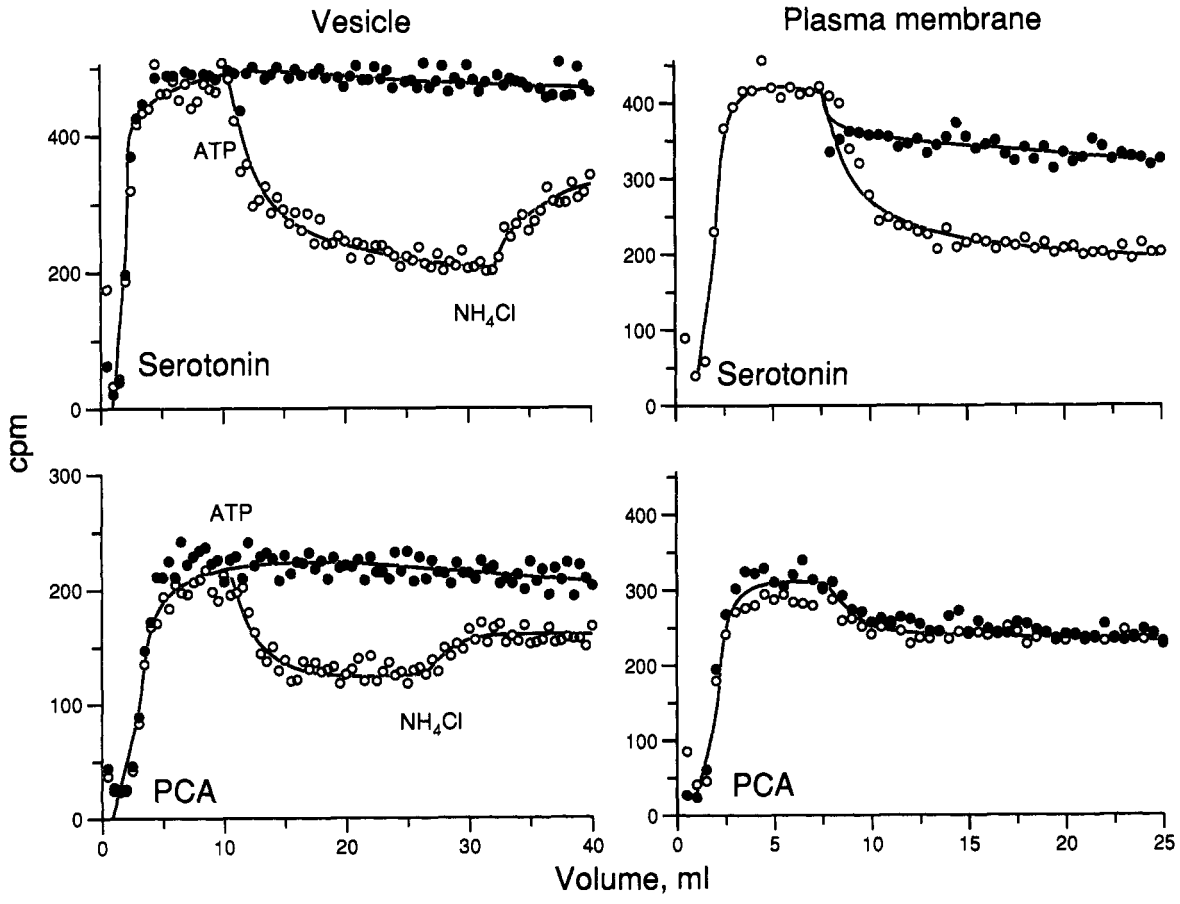

FIGURE 9: Flow dialysis measurements of [^{3}H]serotonin and [^{3}H]PCA accumulation. Flow dialysis measurements were performed as described under Experimental Procedures using the following protocol. Vesicular amine transporter (left panels): 0.1 μM [^{3}H]serotonin (upper left) or 0.07 μM [^{3}H]PCA (lower left) was added to a suspension of 100 μg of chromaffin granule membrane vesicles in 200 μL of 0.3 M sucrose containing 10 mM K-EPPS, pH 8.5, 2.5 mM $MgSO_4$, and 5 mM KCl in the upper chamber of the flow dialysis apparatus. 5 mM ATP (open circles) was added to the suspension in the upper chamber 9.5 min after addition of radiolabel, and after a further 12–15 min, NH_4Cl was added (final concentration, 20 mM) to dissipate ΔpH. Filled circles show a control experiment with no ATP addition. Plasma membrane transporter (right panels):0.1 μM [^{3}H]serotonin (upper right) or 0.07 μM [^{3}H]PCA (lower right) was added to 190 μL of 0.2 M NaCl containing 10 mM lithium phosphate, pH 6.7, and 1 mM $MgSO_4$ in the presence (filled circles) or absence (open circles) of 1 μM imipramine in the upper chamber of the flow dialysis apparatus. After 5 min, 5 μL (100 μg) of a suspension of platelet plasma membrane vesicles equilibrated in 133 mM K_2SO_4 containing 10 mM lithium phosphate, pH 6.7, and 1 mM $MgSO_4$ was added to the upper chamber.

probably reflects the difference between the K_D for equilibrium binding of PCA and the K_M for PCA transport. For serotonin also the K_M for transport is lower than the K_D measured by inhibition of imipramine binding (Talvenheimo et al., 1979). Characteristic of carrier-mediated transport, the rate of serotonin translocation is faster than other steps in the transport cycle. This leads to a decrease in K_M relative to K_D that is dependent on the ratio of rate constants for the substrate translocation step and the rate-limiting step (Marcusson et al., 1986; Talvenheimo et al., 1979). For serotonin, the difference between K_M and K_D is 5–15-fold. Assuming that the rate-limiting step in transport is the same for serotonin and PCA, the 64-fold difference between the K_I values for PCA inhibition of binding and transport (Figures 1, 2, and 4) suggests that translocation of PCA is at least as fast, if not faster, than for serotonin.

PCA may also be a substrate for the vesicular amine transporter, although the results are less compelling. There is clearly competition with serotonin for transport into chromaffin granule membrane vesicles (Figure 5), and at higher concentrations, PCA stimulates [^{3}H]serotonin efflux from these vesicles, but the concentration of PCA required to stimulate efflux is also sufficient to dissipate the ΔpH (generated by the vacuolar ATPase) which drives serotonin accumulation (Figure 8). Furthermore, PCA is more potent than serotonin at inducing efflux (Figure 6), suggesting that, aside from its ability to bind to the substrate site, PCA is a permeant weak base that causes efflux by increasing intravesicular pH. Maron et al. (1983) demonstrated that [^{3}H]-serotonin efflux from chromaffin granule membrane vesicles was exquisitely sensitive to internal pH, as if internal H^+ ions competed with internal substrate for the transporter. This competition by H^+ ions is likely to account for the relative insensitivity of [^{3}H]serotonin efflux to low concentrations of external serotonin or PCA. Accordingly, internal pH, rather than the availability of external substrate, would determine efflux rates.

Another indication that PCA rapidly permeates by nonionic diffusion is the observation (Figure 9) that [^{3}H]PCA is accumulated within chromaffin granule membrane vesicles but not plasma membrane vesicles. Accumulation of permeant weak bases in membrane vesicles is limited by the transmembrane pH difference (ΔpH, inside acid). No ΔpH is imposed in plasma membrane vesicles, and no accumulation is observed. PCA is accumulated within chromaffin granule membrane vesicles in response to ATP-driven H^+ pumping, but serotonin is accumulated to higher steady-state levels. The higher accumulation of serotonin most likely reflects driving forces available for accumulation of substrates that are not freely permeant. For each substrate molecule transported by the vesicular amine transporter, two H^+ ions leave the vesicle, and the overall reaction leads to efflux of one

positive charge (Njus et al., 1986). Thus, both the ΔpH and the $\Delta\psi$, generated by the vacuolar ATPase, provide energy for substrate accumulation. In contrast, nonionic diffusion of permeant weak bases is driven by the formal efflux of one H^+ ion for each protonated amine molecule taken up in an electroneutral process. The steady-state accumulation of PCA is less than that of serotonin and close to that expected of a permeant weak base, suggesting that nonionic diffusion represents the major pathway for its transport across the membrane. A further indication of the permeant nature of PCA is the inability to detect accumulated [^{3}H]PCA by a filtration assay (Table II). This indicates that PCA can leak out of the membrane vesicles in the short time it takes to filter and wash the vesicles.

As a permeant inhibitor of the vesicular amine transporter, PCA is expected to lead to loss of vesicular serotonin in the same way that tyramine, another permeant substrate, releases catecholamines (Knoth et al., 1984). PCA inhibits influx effectively, but its lower accumulation should decrease its ability to compete with serotonin for efflux. At higher concentrations, PCA additionally causes more rapid efflux by dissipating ΔpH, as noted above. At the plasma membrane, PCA can stimulate serotonin efflux by two mechanisms: (1) exchange via the serotonin transporter (Figures 3 and 4, Table I) and (2) dissipation of driving ion gradients. If PCA is a substrate avidly transported by the serotonin transporter, then it must alternately enter the cell via the transporter and leave by passive diffusion. On each cycle, Na^+ and Cl^- are expected to enter the cell and K^+ to exit, leading to dissipation of the driving forces for serotonin accumulation. In addition, the dissipation of transmembrane ion gradients may tax energy metabolism in serotonergic nerve terminals, with a resulting fall in ATP and rise in Ca^{2+} concentrations. The extent to which these consequences contribute to the neurotoxicity of PCA remains a subject for further investigation.

The ability of serotonin transport inhibitors to block the acute and long-term decrease in brain serotonin caused by PCA has never been well explained. One explanation, that the transporter is required for entry of PCA into the cell, is rendered unlikely by the results presented here. The high passive permeability of secretory vesicles and plasma membranes to PCA suggests that lipid bilayers to not present a major barrier to PCA diffusion. A more likely explanation is that the inhibitors block serotonin/PCA exchange and PCA-mediated dissipation of ion gradients.

These mechanisms for PCA-induced serotonin release may also apply to amphetamine and its other congeners. Previous work (Rudnick & Wall, 1992a) indicates that MDMA inhibits serotonin transport and imipramine binding and stimulates serotonin efflux from plasma membrane vesicles. In chromaffin granule membrane vesicles, MDMA inhibits serotonin influx and, at higher concentrations, dissipates ΔpH and stimulates efflux. The similarities between PCA and MDMA suggests that all serotonin-releasing amphetamines, and perhaps all amphetamines, release endogenous biogenic amines by specific interactions with plasma membrane and vesicular amine transporters.

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Registry No. PCA, 64-12-0; serotonin, 50-67-9.